

Human Cytokine Response to *ex vivo* Amyloid- β Stimulation is Mediated by Genetic Factors

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Through its ability to induce the enhanced release and production of cytokines, amyloid- β is responsible for the chronic inflammatory response that contributes to Alzheimer's disease (AD). Determining whether the response of monocytes to amyloid- β stimulation is under genetic control may help understand the basis of why some people are more prone to develop neuronal degeneration than others. In the current study we investigated the heritability of the cytokine (IL-10, IL-6, IL-1 β , IL-1ra, TNF- α) production capacity upon *ex vivo* stimulation with amyloid- β in whole blood samples of 222 twins and 85 singleton siblings from 139 extended twin families. It was found that individual differences in amyloid- β -induced cytokine production capacity are to a large extent of genetic origin, with heritability estimates ranging from 55% (IL-1 β) to 68% (IL-6). We conclude that genes influencing amyloid- β -induced cytokine response may provide clues to the progression of AD pathology.

Senile plaques constitute the key pathological feature of Alzheimer's disease (AD) and are composed of insoluble amyloid- β (A β), activated microglia, astrocytes, and degenerating neurons. The accumulation of inflammatory microglia at sites of A β deposition significantly contributes to neuronal degeneration (McGeer & McGeer, 1995). A β is able to initiate the accumulation and activation of microglia by triggering the complement cascade and induce enhanced release and production of cytokines (Bornemann et al., 2001; Shen & Meri, 2003). A β , itself, may induce a chronic inflammatory response contributing to both further plaque development and neuronal degeneration, detectable in reactive microglia within senile plaques.

In twins, the concordance with diagnosed AD (up to death) has been found to be two- to threefold greater for monozygotic (MZ) than for dizygotic (DZ) pairs, indicating strong genetic influences on the presence of AD (Gatz et al., 1997). Also the age of

onset of AD has been shown to be under genetic influence (Pedersen et al., 2001). The inflammatory response to the presence of A β in the brain could underly this heritable component in AD. In the current twin study we therefore investigated whether production capacity of cytokines upon stimulation with A β is a heritable characteristic.

Microglia are derived from monocytic lineage and arrive in the central nervous system (CNS) late in embryonic development. Based on their lineages and phenotype, microglial cells are generally accepted as the macrophages of the CNS (Davis et al., 1992). Hence, peripheral monocytes may be a useful model to mimic the effects of A β peptides on human microglia function in the brain. To determine the magnitude of the heritability of the production capacity of cytokines upon stimulation with A β , we examined in a sample of MZ and DZ twin pairs and their siblings the cytokine profiles upon *ex vivo* stimulation of whole blood samples with lipopolysaccharide (LPS) and A β . This design allows the determination of the extent to which interindividual differences in A β -stimulated cytokine production capacity can be ascribed to genetic differences between individuals. MZ twins share all, or nearly all (Martin et al., 1997), of their genetic material. DZ twins and siblings share on average 50% of their segregating genes. If resemblances (often expressed as correlations) in MZ pairs are larger than in DZ pairs, this is a first indication that genetic influences are of importance (Martin et al., 1997; Posthuma et al., 2004). The use of the so-called 'extended' twin design (including twins and their siblings) ensures relative high statistical power to detect sources of nongenetic, environmental variation that are shared by family members (Posthuma & Boomsma, 2000).

Received 26 January, 2005; accepted 2 February, 2005.

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Previously we have shown that individual differences in the cytokine production capacity of peripheral monocytes upon lipopolysaccharide (LPS) stimulation can be explained by genetic variation (de Craen et al., 2005; Westendorp et al., 1997). Here we examine whether such is also the case when cells are stimulated by both LPS and A β .

Subjects and Methods

Subjects and Procedure

This study is part of a larger study on the genetics of adult brain function (Posthuma et al., 2001). Twins and their siblings were invited for an additional visit to the lab for blood extraction. Three hundred and seven twins and their siblings from 139 families participated. The age range was 15.6 to 68.7 years (mean age = 38.7, $SD = 12.5$). Forty-six per cent were males, and 54% were females. The total sample included 41 MZ pairs, 52 DZ pairs, and one family consisting of trizygotic triplets. In addition, 85 siblings participated and 33 single twins (who participated with a nontwin sibling). Zygosity of same-sex twins was determined by typing highly polymorphic genetic markers. Fasting blood samples were drawn in the morning before 11.00 am. All subjects gave informed consent and received a breakfast after donating their blood sample. The study was approved by the Medical Ethical Committee of the Vrije Universiteit Amsterdam. All participants provided written informed consent.

Cytokine Production Capacity

Cytokine production capacity was assessed with an *ex vivo* whole blood assay, as described previously with small alterations (de Craen et al., in press; van der Linden et al., 1998). In short, heparinized whole blood samples were first diluted fivefold with RPMI-1640 (Gibco Life technologies). Then, samples were stimulated with either 10 ng/ml LPS (from *Escherichia coli* 0111, Difco Laboratories) alone or 10 ng/ml LPS in combination with 50 U/ml Ab 1-42 fibrils (Bachem) and incubated for 24 hours at 37°C with 5% CO₂. The amyloid was fibrillar as shown by green birefringence in a polarizing microscope after Congo red staining. After centrifugation, the supernatants were stored at -80°C until assaying for Interleukin-1 β (IL-1 β), Interleukin-1ra (IL-1ra), Interleukin-6 (IL-6), Interleukin-10 (IL-10), and Tumor Necrosis Factor- α (TNF- α), using standard ELISA techniques according to the manufacturers' guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands).

Stimulation of unactivated monocytes with A β does not result in enhanced cytokine release. However, A β induces increased release of cytokines from LPS-activated human monocytes (Lorton et al., 1996). Therefore, to assess the individual A β -induced cytokine response the level of cytokine production upon LPS alone was subtracted from production upon LPS plus A β stimulation.

Statistical Analysis

Five subjects were excluded from the analyses: the determination of the cytokine profile failed due to technical problems in 2 subjects, and 3 subjects had TNF- α concentrations of more than 100 pg/ml under unstimulated conditions. The distribution of each cytokine was inspected for outliers, which were subsequently eliminated from the analyses (3 outliers for IL-1 β , 2 for IL-6, and 1 for TNF- α). We tested for heterogeneity across zygosity and sex with respect to means and variances. In addition, the effect of age on the observed cytokine production scores was included as a covariate.

Genetic Analyses

The data were first summarized into correlations for MZ and DZ twin and sibling pairs. Next, the variation in cytokine response upon A β stimulation was decomposed into additive genetic variation (A), shared environmental variation (C) or nonshared environmental variation (E; Boomsma et al., 2002; Posthuma et al., 2004). Shared environmental variation by definition included all environmental sources of variation that twins and siblings from the same family share, while nonshared environmental variation refers to the environmental variation that is unique for an individual and that is typically not shared with other family members. For DZ twin and sibpairs similarity in shared environmental influences is 100% and similarity of additive genetic influences is 50%. There was by definition no similarity in nonshared environmental influences. For MZ twin pairs similarities of additive genetic and shared environmental influences were fixed at 100%. Thus, the expectation for the total variance is $A + C + E$, the expectation for the covariance between MZ twins is $A + C$, and the expectation for DZ twins/sibpairs is $\frac{1}{2}A + C$. Heritability is calculated as the proportional contribution of genetic variation to the total observed variation. All analyses were carried out using the statistical software package Mx (Neale, 1997). Estimation of parameters was obtained by normal theory maximum likelihood. Testing of the significance of A and C was done by constraining them at zero and comparing the resulting fit of the model using likelihood ratio tests (Neale & Cardon, 1992).

Results

The distributions of A β induced cytokine production capacity is graphically represented in Figure 1. The distribution of the production capacity is wide, representing both up and down regulation of individual samples.

Table 1 shows significant upregulation by A β for IL-1 β and significant downregulation for IL-1ra, IL-10, IL-6, and TNF- α . No differences in variances or means between MZ twins, DZ twins and sibling pairs were found. Also, no such differences were found between males and females except for a mean difference in IL-10 production. Men had a higher IL-10 production com-

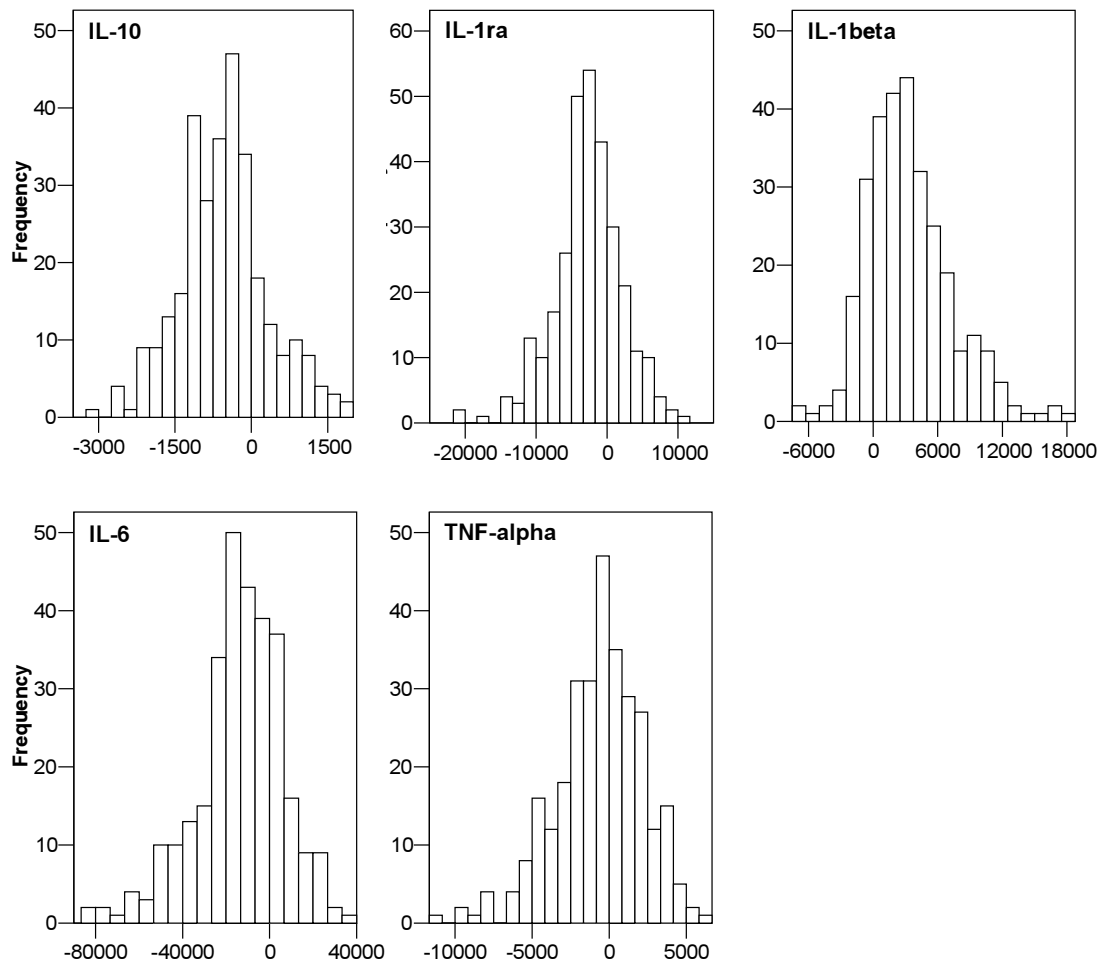


Figure 1

Histograms of the production of cytokines (in pg/ml) upon stimulation of Aβ, calculated by subtracting the cytokine level after LPS stimulation from the cytokine level after subsequent amyloid-β stimulation.

pared to women (+282.71 pg/ml; see Table 1). There was no association with age for any of the cytokines. The difference between cytokine production after LPS stimulation and cytokine production after Aβ stimulation was statistically different ($p < .001$) from zero for all five cytokines.

Maximum likelihood estimates of twin and sibling correlations are given in Table 2. No differences between DZ twin correlations and sibling correlations were found. MZ correlations ranged from .36 for Aβ-induced regulation of TNF-α levels to .65 for Aβ-induced regulation of IL-6 levels. DZ correlations were lower than the MZ correlation, indicating the presence of genetic factors in the Aβ-induced cytokine response.

Decomposition of the variation into additive genetic, shared environmental and nonshared environmental variation indicated that variation in Aβ induced regulation of IL-10, IL-1β and IL-6 variation was caused by additive genetic sources and nonshared environmental sources. Heritabilities of IL-10 were estimated at 58%, for IL-1β at 55% and for IL-6 at 67%. Genetic and shared environmental sources of

variation could not be distinguished between for IL-1ra and TNF-α, although the poor fit of the model that included only nonshared environmental sources of variation indicates that familial (i.e., genetic or shared environmental) sources are important.

Table 1

Sample Descriptives

		N	Mean (pg/ml)	SD
Males (Mean age 38.29 years; SD 12.30)	Aβ-induced production of IL-10	139	-351	970
	Aβ-induced production of IL-1ra	139	-2497	4774
	Aβ-induced production of IL-1β	135	2982	4355
	Aβ-induced production of IL-6	137	-14,716	21,375
	Aβ-induced production of TNF-α	138	-746	3002
Females (Mean age 39.08 years; SD 12.63)	Aβ-induced production of IL-10	163	-718	738
	Aβ-induced production of IL-1ra	163	-2725	4810
	Aβ-induced production of IL-1β	163	3639	3642
	Aβ-induced production of IL-6	163	-13,012	19,711
	Aβ-induced production of TNF-α	163	-585	2702

Note: Amyloid-β production capacity was calculated by subtracting the cytokine level after LPS stimulation from the cytokine level after amyloid-β stimulation.

Table 2Twin and Sibpair Correlations (*r*) for A β -Induced Cytokine Production

	<i>N</i> (individuals)	MZ pairs	DZ/ sibpairs	<i>r</i> _{mz}	<i>r</i> _{dz/sib}
IL-10	302	41	192	.58	.28
IL-1ra	302	41	192	.39	.29
IL-1 β	298	40	186	.55	.27
IL-6	300	41	189	.65	.39
TNF- α	301	41	190	.36	.24

Note: DZ/sibpairs include all possible (nonindependent) DZ/sibpairings within a family. For example a family consisting of DZ twins and three additional siblings forms 10 DZ/sibpairs.

In all cases, a model that included only nonshared environmental influences provided a poor fit to the data, as indicated by a significant *p*-value in the E model (Table 3).

Discussion

The effects of A β on additional cytokine release from *ex vivo* LPS-stimulated whole blood samples show large individual differences. These differences were found to be of genetic origin to a large extent, with heritability estimates ranging from 55% (IL-1 β) to 68% (IL-6). This implies that monocytes and possibly also microglia have an innate cytokine response to the presence of A β . In other words, the chronic inflammatory

response to the presence of A β depends on an individual's genetic background and may thus explain why some individuals are more prone to develop reactive microglia and continue on to develop AD than others.

In the past two decades several genes related to the onset and progression of symptoms in AD have been identified, such as ApoE, Presenilin-1, Presenilin-2, Amyloid Precursor Protein (Corder et al., 1993; Goate et al., 1991; Levy-Lahad et al., 1995; Rogaev et al., 1995; Selkoe, 1996; Sherrington et al., 1995). Together these genes account for roughly 10% of AD prevalence. Furthermore, genetic variants within the IL-1a, IL-1 β and IL-1rn genes have been associated/related to age of onset and progression of symptoms of AD (Alvarez et al., 2002; Combarros et al., 2002; Griffin & Mrak, 2002; Licastro et al., 2003; Sciacca et al., 2003). Possibly these variants affect the innate production capacity towards a pro-inflammatory profile.

Cytokines play critical roles in the development and progression of AD (Mrak et al., 1995), not only as a consequence of the disease, but also as a precursor to it. The presence of A β stimulates microglia, astrocytes and oligodendrocytes to secrete proinflammatory cytokines, a very early step in the development of AD (Mott et al., 2004; Tan et al., 1999). Recently, we have shown that the production of cytokines in a LPS-stimulated whole blood assay is under strict genetic control (De Craen et al., in press). The present study expands this evidence by showing that in healthy, non-AD human subjects of middle age, the A β -induced inflammatory response is also under genetic control.

It has been suggested that chronic inflammatory responses contribute to the development of AD pathology (Akiyama et al., 2000; Eikelenboom & van Gool, 2004). Here we propose that the heritable component in the rate of AD progression is partly explained by the innate cytokine production capacity of monocyte-derived cells. Our results warrant further investigation of LPS/A β responses and variation in cytokine and cytokine receptor genes in relation to AD age of onset and progression.

Acknowledgments

This study was carried out with financial support from the Universitair Stimulerings Fonds (grant number 96/22), the Human Frontiers Science Program (grant number rg0154/1998-B), and the Hersentstichting Nederland (8F00.06). D. Posthuma was supported by the GenomeEUtwin project (European Union Contract No. QLG2-CT-2002-01254). We thank Corine de Koning-Treurniet, Marja Kersbergen-van Oostrom, and Margot van Schie-Troost for whole blood stimulations and cytokine determinations.

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Table 3

Fit results from models where the variance of A β -induced cytokine production is decomposed into additive genetic variance (A), shared environmental variance (C) and nonshared environmental variance (E).

	Tested	Versus	<i>p</i> -value	%A	%C	%E
IL-10	ACE	Null	.48	58	0	42
	AE	ACE	1.00	58	—	42
	CE	ACE	.02			
	E	AE	.00			
IL-1ra	ACE	Null	.65	21	19	60
	AE	ACE	.30	44	—	56
	CE	ACE	.42	—	32	68
	E	AE	.00			
IL-1 β	ACE	Null	.40	55	0	45
	AE	ACE	1.00	55	—	45
	CE	ACE	.02			
	E	AE	.00			
IL-6	ACE	Null	.65	52	13	35
	AE	ACE	.38	67	—	33
	CE	ACE	.02			
	E	AE	.00			
TNF- α	ACE	Null	.22	25	11	64
	AE	ACE	.54	42	—	58
	CE	ACE	.46	—	25	75
	E	AE	.00			

Note: *The ACE models were all tested against a saturated model in which the variance was not decomposed into A, C and E (i.e., the 'null model').

A nonsignificant *p*-value denotes a nonsignificant worsening of the fit. Best model in bold.

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